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ENTITLED Studies on Formation, Regeneration and Manipulation of Protoplasts from *Caldariomyces fumago*

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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STUDIES ON FORMATION, REGENERATION AND MANIPULATION OF PROTOPLASTS FROM CALDARIOMYCES FUMAGO

BY

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PG#

I. Abstract

ABSTRACT:

We have recently investigated the production and regeneration of protoplasts formed from Caldariomyces fumago, a filamentous fungi, and have tried a few transformations for a chloroperoxidase negative mutation. The formation of protoplasts has been successfully done by homogenizing the mycelia followed by digestion in NovoZym 234. Recovery is good but variable, and has ranged from 5×10^7 to 6.5×10^9 total cells using a banding method. The digest is enhanced by homogenization and typically run for three hours. The banding recovery occasionally does not work well as some small mycelial fragments often contaminate the sample. However, this recovery does provide a good concentration and recovery for the cells.

Several regeneration studies done indicate that glucose is a better substrate than fructose and that the minimal sugar concentration for correct osmotic conditions is 100g/l. Regeneration appears to be better in softer agar, consequently, overlays are beneficial. Incubations before plating have given mixed results. It appears that only incubation in the glucose-100 media for about an hour has favorable results. Increased time of incubation tends to lower regeneration. Lower temperatures also appear

to be helpful, however, the benefit of better regeneration may be negated by the time loss as growth is significantly slower. Optimal conditions for regeneration therefore appear to be incubation in glucose-100 for one to two hours followed by plating onto or with an overlay of glucose agar.

Besides the studies on plating media, incubation solutions and temperature, the storage regeneration of protoplasts was also examined. Stored at 4 degrees Celsius, in isotonic or KCl buffer, the cells lose about 7% viability per day. In addition to this , an ultraviolet sensitivity study has been done to determine the viability of treated cells as a pilot study for possible use of UV mutagenesis.

Attempts in isolation of the C. fumago chloroperoxidase negative mutant through transformation and subsequent screening have not been successful. The developed colormetric test is incapable of screening the very large number of colonies required for this rare gene "knock-out". New methods based on either a selection marker or a better screen for chloroperoxidase need to be developed. We will therefore examine the future possibilities as well as the results in detail.

INTRODUCTION:

The development of expression systems has traditionally been centered upon the use of Escherichia coli or bakers yeast, Saccharomyces cerevisiae. Recent investigation of filamentous fungi has revealed promising new expression systems with important advantages. Filamentous fungi are noted for their high protein secretion levels and are often able to post-transcriptionally modify proteins (1) . Of the filamentous fungi, perhaps the most investigated species is Aspergillus nidulans and the secretion of human plasminogen activator has been reported (2) .

As noted by Upshall in his recent article in BioTechniques, the development of several fungal expression systems will provide valuable alternatives in the eventual production of mammalian proteins as there are different advantages to each system, depending on the desired product (1). One possible new expression system involves the use of Caldariomyces fumago (C. fumago). This fungus secretes a native protein, chloroperoxidase, in quantities of up to 500 mg/liter and can be adapted to a continuous fermenter. (3)

Recent work in the laboratory of Dr. Lowell Hager by Steven Blanke has demonstrated the feasibility of maintaining an actively secreting culture for up to six months (4). The C. fumago grows as a uniform layer on the wall of the rotating test carbide. The media can therefore be removed or added as needed from the culture. A continuous flow system with the properly adjusted flowrates of media and oxygen can easily maintain a culture for months and is presently being investigated. C. fumago grows well on a minimal defined media, and aeration in this set-up only requires slow rotation of the culture carbide. The system is thus ideal as upkeep of the culture is inexpensive both in energy consumption and materials. For possible industrial usage, the adaptability of the C. fumago to a continuous batch recovery represents a significant economic advantage over other expression systems for heterologous proteins.

In addition to heterologous proteins, the expression system can be used to study the enzyme, chloroperoxidase, in greater detail as it would be ideal for site-specific mutagenesis. Since the protein is native to C. fumago, this should eliminate any difficulties in the proper post-transcriptional modification and secretion of the mutant proteins (5).

Initial interest in *C. fumago* was the result of the fungus' ability to utilize inorganic chloride and subsequent production of caldaryomycin, an antibiotic (6). Chloroperoxidase was discovered to be the halogenating enzyme responsible and extensive characterization has been done in the Hager laboratory. Chloroperoxidase has been determined to contain a ferriprotoporphyrin IX prosthetic group and has activity in a low pH range (7). It has been shown by Hager, *et.al*. that the enzyme can halogenate β-keto acids, cyclic β-diketones, and substituted phenols with chloride, bromide and iodide ions (8). They also developed a sensitive assay for chloroperoxidase which involves the conversion of monochlorodimedon to the dichloro state with the subsequent loss of absorbance at 274 nm.

Besides characterization of the chloroperoxidase enzyme, work with *Caldariomyces fumago* has not been extensive in any respect. A review of the literature existing on mycological studies yields a few details. *C. fumago* is known to grow on plants in greenhouses, and was first identified in Northern Europe and classified as *Fumago vagans* (6).
Fumago vagans was defined as polymorphic, sooty mould and thought to be an imperfect state of *Capnodium* by Montagne in 1849 (9). A detailed

description of *C. fumago* is provided by Goos and Pirozyski (10) and some photographs of colonies on agar are included in the pictorial appendix. A 1965 study by R. J. Friend concluded that the classification of *Fumago vagans* was incorrect as the dark sooty moulds were often a composite of different varieties as opposed to being polymorphic (9). The present classification of *C. fumago* is as follows: Order -- Moniliales, class -- Hyphomycetes, sub-division -- Deuteromycotina, division -- Eumycota, kingdom -- Fungi (11).

Two studies, one on metabolism, the other on growth, have been done on *C. fumago*. A study in 1963 by Ramachandran and Gottlieb concluded that *C. fumago* only carried out aerobic respiration. The study was based on growing the fungus in a defined glucose minimal media. Glucose was shown to be catabolized to gluconate with the subsequent release of hydrogen peroxide. The gluconate was then transformed to 2-keto gluconate. Addition of catalase to the media greatly increased the oxidation rates by breaking down the peroxide produced in the first step. Ramachandran concluded that *C. fumago* utilized the Entner-Doudoroff and hexose monophosphate shunt to the Kreb cycle and probably the electron transport system with cytochromes. (12)

A different study by Pickard in 1981 explored the effects of media on chloroperoxidase production by ten different stains of C. fumago. Pickard examined chloroperoxidase levels, pigmentation and pH in the media. He concluded that production on a glucose-malt media (40 and 20 g/l) reached a maximum on twelve days of growth, presumably when the glucose had been exhausted and the fungus switched over to the malt. Growth in fructose media created the same high level of chloroperoxidase at six days growth and a lower pigmentation level. Pickard's results indicate that the ATCC 16373 and CMI 89362 strains produced the most chloroperoxidase, the highest levels being in fructose or inulin media.

The cultures grew well in most carbon sources, only citrate and cellulose being poor carbon sources (13).

Since little other work exists on C. fumago, all present transformation procedures are based on work with other filamentous fungi, most notably, Aspergillus nidulans. In order to transform a fungal cell, the wall must be removed. Subsequently, the formation of protoplasts is the initial step required. Protoplasts have been formed for several species of filamentous fungi, yeast, and plant cells such as root tips of tomato seedlings (14). Two extensive reviews of fungal protoplasts and their uses have been complied by Peberdy, 1979 (15) and Villanueva and Acha, 1971 (16). Villanueva provides the distinction between the terms, "protoplast" and "spheroplast" by defining a protoplast to be the complete removal of the cell wall and a spheroplast as a structure with a partial cell wall left.

In the formation of these structures, there are two important factors: the digestive enzyme used and the osmotic stablizer for the fragile membrane sacs. Villanueva mentions the use of snail (Heliz pomatia) digestive juice by Giaja in 1914. The effectiveness of the juice has been identified as the presence of β-glucanase. Other digestive preparations have included microbial enzymes from Bacillus and Streptomyces (16). Protoplasts have been sucessfully formed from Neurospora crassa, Aspergillus, Candida utilis and Phytophthora to name a few. On Saccharomyces fragilis, the addition of β-mercaptoethanol greatly increased the effectiveness of the digestion, possibly due to reduction of disulfide bonds (16).

Common osmotic stabilizers are mannitol, sorbital, sucrose, potassium chloride and magnesium sulfate. The pH of the solutions is typically in the range of 5.8 to 6.8 at concentrations varying from 0.3M to 1.0M (16). Recovery of the protoplasts formed by digestion of the hyphae is typically achieved by filtering the protoplasts from the debris in either a sintered glass funnel or through nylon cloth (16, 17). The protoplasts are then centrifuged and washed. Work by Peberdy and Issacs in 1976 demonstrated that protoplasts from *Aspergillus nidulans* vacuolated in 0.6M magnesium sulfate (18). Subsequently, the protoplasts can be recovered by a banding method. The digestion mixture is placed in magnesium sulfate with a lighter buffer on top and centrifuged, resulting in the formation of a sharp band at the interface (19).

Regeneration values for the recovered protoplasts are variable. Perberdy gives the typical reversion as fifty percent but values for yeast are fifty to seventy percent for *Saccharomyces cerevisiae* and ninety percent for *Schizosaccharomyces pombe* (15). There are several factors which affect regeneration such as the media (whether liquid or agar solidified), the osmotic conditions, and temperature.

Protoplasts have been valuable to investigators by providing a preparative method for recovery of cell organelles. They can also be used in the preparation of cell-free extracts, and to study the biosynthesis of cell walls (16). Additionally, fusion studies and hybridization can be done as well as transformation (15). Protoplasts provide new and interesting possibilities in genetic manipulation.

There is a large amount of recent work with transformation of protoplasts. Sucessful transformations in Aspergillus nidulans, Neurospora crassa, Cochliobolus heterostrophus, and Podospora anserina have been reported. (19, 20, 21, 22). The typical method of transformation involves the incubation of the protoplasts with DNA in an osmotic buffer with $CaCl_2$, typically for twenty to thirty minutes. This is followed by the addition of polyethylene glycol (PEG), thirty to sixty percent, for roughly the same time span followed by plating onto selective media.

The typical amount of DNA used ranges from about 1 to 50 µg. Most studies do not show an optimum amount. Typically, it appears that the more DNA used, the better the transformation. However, one study by Dhawale, et.al. on Neurospora crassa indicates a plateau after 20 µg (20). Linear DNA with homologous regions is better for transformation via integration than circular plasmids. This was shown in yeast by Orr-Weaver, et.al. (23).

The treatment in $CaCl_2$ is normally used to induce the membrane to allow DNA uptake. The PEG is added to bring the DNA onto the cells so that uptake is enhanced. In Dhawale's study on Neurospora, increasing the incubation in PEG to one hour enhanced transformation (20). The transformation frequencies reported vary from Tilburn who reports 25 transformants / microgram DNA / $2X10^5$ cells in Aspergillus to one to three / fifty micrograms of DNA / $3 X 10^6$ cells with Cochliobolus heterostrophus by Turgeon (21). Transformation frequencies reported are also affected by whether the researchers report the total number of transformants or only the stable ones.

Abortive transformants are discussed by Turner and Ballance as colonies incapable of further growth due to introduced DNA not achieving stable integration. Homologous recombination can occur as a type I,II, or III integration. Type I involves a single crossover event resulting in the complete integration of the plasmid. Type II occurs as a double crossover and occurs at a site other than that of the locus of the host gene. Type III is typically the least common and occurs as a double crossover at the same gene site. Transformation can also result in the multiple insertion of the introduced gene (24).

The goal of this study is to acheive a type III integration in C. fumago using the chloroperoxidase gene which has been cloned and sequenced (3). Using a plasmid construct which is a pUC18 vector with homologous flanking regions and the deleted chloroperoxidase structural region, we hope to "knock out" the native gene and create a chloroperoxidase negative mutant of C. fumago. This mutation would be valuable as it would of course demonstrate DNA intake and site-specific integration. Additionally, the phenotype would provide valuable insights into the possible role of the enzyme in the secondary production of several observed products such as the black pigmentation and the sticky secreted polysaccharide.

In order to produce this mutation, several procedures had to be adapted to C. fumago from other work. The production of protoplasts was achieved by use of NovoZym 234 which is a commercially produced enzymatic mixture of cellulase, chitinase, laminerinase, proteinase, and xylanase (25). Recovery is achieved by centrifugation and banding. Some optimization of production and recovery has been accomplished. The regeneration of the protoplasts is very important and has been extensively studied with several variables. At the present, no chloroperoxidase negative mutation has been isolated, the major setback lying in the lack of of an efficient screening method.

Two screen methods have been used in this study. The first screening method involves the use of a phenol red / KBr detection system. Bromination of phenol red to bromophenol blue has been observed in the uterus of pregnant dogfish, Squalus acanthias (26) . The system developed is a colormetric examination for chloroperoxidase which will halogenate the phenol red. The reaction is buffered at a pH of 4.0 in which phenol red is yellow and bromophenol blue is the same color as its name. The reaction is catalyzed by hydrogen peroxide. (See also figure 5) This assay has been used in agar, the phenol red and potassium bromide incorporated. After fungal colonies become established, the plates are flooded with peroxide and visually scored.

The assay has also been used in a system with microtiter plates which is discussed in the "Materials and Methods." The second screen is based on chloroperoxidase conversion of iodide to the triiodide complex which has strong toxic effects. Only a pilot study has been done with this screen and is examined in the discussion of this paper.

Since no specific chloroperoxidase deficient mutation has been isolated to date, the following results and discussion will therefore center upon the production, recovery, regeneration and storage of protoplasts. All the attempted transformations will also be evaluated and possible future stratagies analyzed.

MATERIALS AND METHODS

C. fumago culture maintainace :

C. fumago cultures were kept on potato agar slants, 60g/l (Difco), and sub-cultured onto a new slant about once every 3-4 weeks. Small inoculums were removed with an inoculation loop and placed into a 50ml Erlenmeyer with 20ml of glucose-malt media, (40g/l glucose, 20g/l malt, 2g/l of KH_2PO_4, $NaNO_3$, and KCL, 1g/l $MgSO_4 \cdot 7H_2O$, and 0.02 g/l $FeSO_4 \cdot 7H_2O$). These small cultures are typically grown for a week and then used to inoculate a large flask (1000ml Erlenmeyer with 200ml glucose-malt media, same as above except 100g/l glucose, 10 g/l malt). Growth to a large culture typically requires seven to ten days. The C. fumago cultures are maintained in a 19 degree Celsius room on a shaker. A culture is deemed fully grown when the media is heavily pigmented and full of large starry clusters, typically with some banding growth on the sides of the flask. Originally, the C. fumago was maintained as a continuous liquid culture (after being started up from a slant) by transferring a few clusters by use of a pipet with a sawed off tip. About six months later, the C. fumago cultures became sickly and grew poorly. Since then, the cultures are maintained by regular transfers from slant to liquid as described above.

Media Growth Studies:

Cultures were grown for comparisons by the following method: a one ml sample of ground inoculum was added to 100ml of media in a cotton plugged 500 ml Erlenmeyer. The samples were grown for about two weeks, visually examined and then spun down in 250ml centrifuge bottles on a swinging bucket rotor (Sorvall HS4) at 4810 X G. The media was decanted and the wet weight of the mycelia determined. Table one gives the medias studied.

TABLE ONE--GROWTH MEDIAS

MEDIA#	SUGAR	NITROGEN SOURCE
1	Glucose	Malt
2	Glucose	Yeast
3	Glucose	Casamino Acids
4	Glucose	None added
5	Fructose	Yeast
6	Fructose	Malt
7	Sucrose	Malt

All medias contain a sugar concentration of 100g/l and the nitrogen source concentration of 10g/l as well as the <u>standard salts:</u>
2g/l of each of the following: KCl, KH_2PO_4, and $NaNO_3$. 1g/l of $MgSO_4 \cdot 7H_2O$, and 0.02 g/l of $FeSO_4 \cdot 7H_2O$.

Protoplast Formation and Isolation :

Protoplasts were formed according to the method described by Tilburn, et.al. (19) with a few changes. Fully grown glucose-malt cultures, typically seven to fourteen days old, are harvested by centrifugation on the Sorvall HS4 rotor at 3079 X G. The dark media is decanted, and the pellet is resuspended into isotonic buffer (0.7M NaCl, 0.2M NaH_2PO_4, pH 5.8) at a ratio of approximately 5 ml to 1 gram. This is then ground for about 2 minutes at a setting of five on a Polytron homogenizer (Brinkmann Instruments), creating an even mycelial suspension. NovoZym 234 (Novo Biolabs) is then added in a concentration of 15mg/g of ground mycelia and the digestion mix placed on a Nutator (Clay-Adams) and allowed to proceed for three hours. Thoroughness of digestion is monitored by microscopic examination, and a good digestion typically shows a dense solution of cells. After the digestion is deemed complete, the mixture is spun down at 6927 X G on the HS4 swinging bucket rotor and resuspended into 1.2M $MgSO_4$, 10mM phosphate, pH 5.8. This is then aliquoted into pre-sterile 50ml centrifuge tubes with a screw cap. 10ml of KCL buffer (0.7M KCL, 10 mM phosphate, pH 5.8) is then carefully layered on top. This mixture is then spun on an IEC clinical centrifuge (Damon/IEC Division) for ten minutes at 1650 x G. A white band forms at the interface, although the relative cleanness is variable as occasionally, some mycelial fragments also float and are suspended immediately beneath the band of protoplasts.

The protoplasts are removed from these tubes with a Pasteur pipet and spun down in 1.5ml microfuge tubes, forming a white-tan pellet. Most protoplasts pellet at a setting of 8160 x G on the Eppendorf microfuge. The cells are then concentrated as desired while resuspending into KCl buffer. Cell counts are done a hemacytometer (Reichert Scientific).

Regeneration Studies:

Regeneration is accomplished by plating the appropriate dilution on agar plates. Table three (pg 18) lists all the various types of media studied. The cells are often incubated in a media for one to three hours (See Table two, pg 18) prior to plating. After plating, cells typically require two days before they are visible under low power in the microscope as a thin, slightly branched strand. After four to five days at room temperature, the colonies are barely visible as fine starry clusters. Regeneration percentages are determined by counting the number of colonies on the plate and dividing by the assumed number plated.

To determine the feasibility of storing protoplasts, a storage study of the cells in two separate buffers, KCl and isotonic, was carried out. The experiment was done as for a regeneration study and the only variable in the study was time of storage, which spanned from day zero to day thirteen. All regeneration in this experiment was done with the standard glucose plates (refer to Table three).

Effects of Ultraviolet Light Upon Protoplast Regeneraton:

To determine the viability of protoplasts after treatment with ultraviolet light, a 254nm lamp (115 volts, 60 Hz, 0.16 amperes) was held 70 cm from the sample which was five mililiters of glucose-malt media with 1 x 10^7 protoplasts in an open petri dish. The dish was agitated slowly by a shaker . 100 µl aliqouts were removed at time intervals of 1,2,4,6,8,10, and 15 minute intervals, to which 900µl of media was added and incubated for one hour before being plated out on color fructose media. The effects of the UV light were determined by comparing the untreated cells regeneration versus the various timed exposures.

TABLE TWO--INCUBATION SOLUTIONS

BUFFER	COMPONENTS
Glucose-50	Glucose , 50g/l, Malt, 20g/l, Salts.
Glucose-100	Glucose, 100g/l, Malt, 10g/l, Salts.
Fructose-100	Fructose, 100g/l, Yeast, 0.1g/l, Salts.
Glucose-KCl-1	Glucose, 50g/l, KCl, 0.35M, 5mM Phospate,Salts.
Glucose-KCl-2	Glucose, 100g/l, KCl, 0.4M, Salts.
Glucose-KCl-3	Glucose, 100g/l, KCl, 0.6M, Salts.
Glucose	Glucose, 100g/l only
Isotonic	0.7M NaCl, 0.2M Phosphate, pH 5.8
KCl	0.7M KCl, 10mM Phosphate, pH 5.8
Sorbital	1.2M Sorbital, 10mM Tris·HCl, pH 7.5

Salts in this table is the same as the standard salts found in table one.

TABLE THREE--REGENERATION MEDIAS

SUGAR	CONC, g/l	NITROGEN SOURCE	%AGAR
Glucose	50.	Malt, 10g/l	2
Glucose	100.*	Malt, 10g/l	2
Glucose	150.	Malt, 10g/l	2
Glucose	200.	Malt, 10g/l	2
Fructose	50.	Malt, 10g/l	2
Fructose	100.**	Malt, 10g/l	2
Fructose	150.	Malt, 10g/l	2
Fructose	200.	Malt, 10g/l	2
Fructose	100.	Yeast, 10g/l	2
Glucose	100.	Malt, 10g/l	1.2 (overlay)
Fructose	100.	Malt, 10g/l	1.5 (overlay)

TABLE THREE CON'T
All medias in the table also contain the following standard salts:
2g/l of each of the following: KCl, KH_2PO_4, and $NaNO_3$. 1g/l of $MgSO_4 \cdot 7H_2O$, and 0.02 g/l of $FeSO_4 \cdot 7H_2O$.

Color Fructose Media:
100g/l fructose, 10g/l malt extract, 2g/l $NaNO_3$, 1g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l of $FeSO_4 \cdot 7H_2O$, 6.6 g/l of KH_2PO_4, 4 g/l of KBr, 20g/l agar and phenol red to sat'd. The KH_2PO_4 concentration is increased to buffer the media. The media is mixed and adjusted to a pH of 4.0 before adding agar or dye. The entire concoction is then autoclaved. Three idential versions of the color media is used in a regeneration study, the only variation being the percent agar, which were 2,3, and 4 percent.

* This is referred to as the standard glucose agar
**This is referred to as the standard fructose agar

Plasmid:

The plasmid used in all transformations is the pUCCF64 which was constructed by Guo-Hua Fang. It consists of a pUC18 vector (2.7 kb) with the ampillicin resistance marker and insert (6.9 kb). The vector is ligated to the 3' end of the insert with a polylinker. The insert consists of 5' and 3' flanking regions to the chloroperoxidase gene. The actual structural gene is deleted between two restriction sites, the Xho I and Nde I sites which spans 910 bp. The restriction map is provided in Figure one of the appendix (27). In all transformations, the DNA is linearized by digestion with Hind III and suspended in water before use.

Transformation Procedures:

Transformations were attempted by using a modified procedure from Tilburn (19). Cells were placed into the transformation buffer which consists of 0.7M KCl, 50mM $CaCl_2$, 10 mM Tris, pH 7.4 . The DNA was added in an equal volume of water and mixed. A quarter volume of PEG solution, (30% polyethylene glycol 6000, 10 mM $CaCl_2$, 10mM Tris HCl, pH 7.5) is added, and the mixture is incubated on ice for twenty minutes. This is followed by a 1:10 dilution by PEG solution and incubated at room temperature for another twenty minutes. The cells are then diluted out into a large volume of incubation media, typically by a ratio of at least 1:50 and allowed to incubate approximately 1-2 hrs. They are then plated on color fructose media or in one case, standard glucose plates.

Screening:

Screening was attempted in two ways. One screen involves the cells being directly plated onto the color fructose media. The colonies are allowed to grow for about ten days at room temperature or sixteen at 15 degrees Centigrade. Screening is done by flooding the plate with a 1:200

dilution of 30% hydrogen peroxide. Color development takes typically three to five minutes and colonies developing a blue halo are deemed chloroperoxidase positive. The second screen involves regeneration on the standard glucose malt media which spans about six days. The colonies are then transferred by toothpicks individually into 300µl well microtiter dishes (Falcon).

The wells are filled with fructose-100 media and allowed to grow one to two weeks before the "toothpick" screen is used. This screen consists of a microtiter plate with white blotter paper attached to the bottom. Saturated phenol red solution with 10g/l KBr, pH 4.0, is placed into each well. Each microtiter colony is screened by stirring the toothpick into the fungus well and then placing it into the corresponding detection well. About 50 to 75µl of hydrogen peroxide (same dilution) is added. The color change is the same as before, and all blue wells are positive for chloroperoxidase.

Another possible screen involves the use of iodide. Media identical to the standard fructose plates is made with the removal of KCl and the addition of KI in varying concentrations of 5, 10, 20, 50 and 83 g/l. The cells are allowed to grow until barely visible to the eye and then a 1:1000 dilution of hydrogen peroxide, 30% added. The formation of the triiodide ion causes the agar media to develop a dark brown color and very young colonies in this region die.

RESULTS AND DISCUSSION

Media Studies:

The growth and visual appearance of the C. fumago depends strongly on the sugar and nitrogen sources used. From the media studies, C. fumago grows well in liquid culture on glucose and fructose when supplemented with a complex nitrogen source, but rather poorly on sucrose with malt. Visually, the cultures are also very different. C. fumago grown in glucose-only resembled that of sucrose malt in that it forms large fuzzy spherical clumps and did not develop to a full culture. Samples grown in glucose-malt were similar in appearance but exhibited much more growth in the time span. The glucose-yeast culture was a much finer suspension and appeared to be a healthier growth. The glucose-casamino acids sample was similar to that with yeast while the fructose-yeast culture

was definitively different. This culture appeared to contain smaller spherical clusters but also produced a very large amount of extracelluar matter which is best described as a viscous green slime. The fructose-malt culture was similar with less additional product.
The following Table four gives the wet weight of mycelia determined. These values are, of course, not very accurate as water retention may vary due to differences in growth formation. However, the values do supply some form of comparision.

It is interesting to note the very different visual forms that C. fumago assumes upon different medias. C. fumago grown on glucose agar is typically a fuzzy grey-green, often with dark concentric ringing. Fungus grown on fructose agar begins similarly, but soon turns blackish and exudes a sticky polysaccharide. This would suggest that there is an entire series of genes which are posssibly controlled together as fructose induces chloroperoxidase formation and this dramatic change in form. (See also photographs in appendix).

TABLE FOUR--WET WT OF MYCELIA

MEDIA TYPE	WET WEIGHT(grams)
Glucose	10.8
Glucose-Caseamino Acids	34.5
Glucose-Yeast	43.3
Sucrose-Malt	9.0
Fructose-Yeast	100.9
Fructose-Malt	49.3

This experiment was run for sixteen days of growth, the weights are averaged between two samples for each type.

Glucose-Malt	15.4
Glucose-Yeast	46.3
Fructose-Yeast	86.2

This data is from a separate experiment, growth was allowed for fourteen days.

Protoplast Formation and Optimization:

In the formation of protoplasts, three variables were examined, specifically, the digestion mixture, the time of digestion and the amount of grinding done on the Polytron. In a pilot experiment, Dr. Hager examined the effects of pre-treating the mycelia with 0.1 M dithiothreitol for one hour before digestion with NovoZym 234. This did not improved protoplast formation.

For the time trials, the digestion was monitored visually, and was checked at one, four and twenty-four hour intervals. Under the scope, the four hour digestion appeared much better than that obtained at one hour. The twenty four hour digest did not appear to have progressed much further. The formed protoplasts also seemed sticky. It was therefore deemed unneccesary to run the digestion overnight.

Grinding time was studied as none, a very short grind of 30 seconds and a long one of 120 seconds. On microscopic examination, the 120 second grind sample exhibited the best digestion followed by the short grind and the untreated. Recovery of protoplasts was done by centrifuging mycelial at 650 X G in a fixed angle rotar (Sorvall GSA) followed by a spin at 7710 X G (Sorvall SS34) and resuspension into 10 ml of isotonic buffer. Results showed that the 120 second sample had the best recovery at 3.75 X 10^6 cells/ml. The 30 second sample had a recovery of 2.0 X 10^6 cells/ml and the untreated at 1.0 X 10^6 cells/ml. This experiment produced the expected results as one would expect a better digestion due to the greatly increased surface area.

Three different methods for recovery were tried. The first method is as described in the preceding paragraph, the second was filtration through a triple filter of glass wool, cotton and cheesecloth. The collected filtrate was then spun down at 7710 X G on a Sorvall SS34 rotor and resuspended into isotonic buffer. The final method is banding as described in "Materials and Methods." Of the three methods, banding was determined to be the most useful as the cells were well concentrated to about 10^7/ml by this step.

Cell yields are highly variable due to the different mycelial weights, degrees of digestion reached by each culture and the total effort expended in collection attempts. Typically, one can recover about one-third more cells if the banding is repeated on the mycelial digest. Collections have therefore ranged from about 5×10^7 to a "bumper" recovery of 6.51×10^9 total cells.

Morphologically, the protoplasts appear to be of two types, nucleated and non-nucleated. The protoplasts without a nucleus appear to be much lighter as they frequently do not spin down and have little regenerative potential. The nucleated protoplasts appear granular under the microscope and are what is mostly recovered in a preparation. As far as the visual resolution in the microscopes has allowed (675X), there appears to be no residual cell wall after the digest.

Regeneration Studies:

Several variables in the regeneration of protoplasts where examined and fall into three catagories; regeneration agar, incubation media and time, and temperature. The experiments were done as a series of six different studies and as regeneration values fluctuate between preparations, values can typically only be compared within the given experiments. The experiments are therefore summarized individually in tables and discussed as such.

The first experiment involves a comparision of three regeneration medias, incubation in glucose-malt versus none, and the effects of a soft agar overlay . The results are as given:

TABLE FIVE--REGENERATION STUDY #1

MEDIA (3% Agar)	REGENERATION VALUES w/ incubation	 w/o incubation
Stnd Glucose*	15%	9%
Stnd Fructose	11%	7%
Color Fructose	6%	2%
Color Fructose+Overlay	11%	4%

TABLE FIVE CON'T

The overlay is fructose-1.5% agar. Number of samples is two per value.
*Note that agar percentages are 3% as opposed to the stnd 2% for all medias.

It is apparent from this study that regeneration on glucose is better than on fructose and both are better than the color media as expected since the phenol red detection system adds quite a bit of toxicity to the media. Incubation appears to improve the regeneration significantly and the soft overlay from this experiment doubles the value on color media.

Study number two repeats the agar overlay onto standard fructose (except 3% agar) with glucose (1.2% agar) after an one hour glucose-malt incubation. The improvement in regeneration here is also significant as a 29% regeneration is acheived versus standard fructose at 5.8%. The third study compares the effects of varying the agar concentration from two to four percent. The experiment is done with the color fructose agar and also compares five different incubation medias as summarized in Table six.

TABLE SIX--REGENERATION STUDY #3

BUFFER (1 hr incubation)	REGENERATION PERCENTAGES 2% AGAR	3% AGAR	4% AGAR
Glucose-100	2.0	1.6	0.4
Glucose-40	0.3	0.2	0.1
Isotonic	0.8	0.3	0.02
KCl	0.7	0.2	0.03
Sorbital	0.4	0.1	0.003

There are two samples per value.

From this data, there is a clear indication that regeneration is favored with lower agar concentrations. The data on the incubation solutions indicate that glucose-100 is most favorable, followed by the isotonic and KCl and lastly by the glucose-40, sorbital. However, the importance of incubation can not be extrapolated from this data as no control of zero incubation was done. One only has here a comparision of the buffers.

The fourth regeneration study explores the osmotic effects of the regeneration agar and the incubation medias. There is a series of eight agars, as specified in Table three. They are four glucose-malt agars with a varying concentration of 50 to 200g/l and four fructose-malt of the same variety. Three different solutions were used for a three hour incubation (Glucose-KCl 1,2 & 3 see Table two), results in Table seven.

TABLE SEVEN--REGENERATION STUDY #4

MEDIA	REGENERATION PERCENTAGES			
(AGAR)	Control	Media 1	Media 2	Media 3
Glucose-50	2.8	2.1	1.4	1.5
Glucose-100	11.1	7.2	3.2	3.1
Glucose-150	12.7	7.1	4.0	3.5
Glucose-200	12.3	6.5	2.4	3.2
Fructose-50	0.5	1.7	1.5	1.5
Fructose-100	7.3	5.8	2.2	3.3
Fructose-150	7.1	5.9	3.5	3.4
Fructose-200	7.9	6.0	3.7	3.3

These results are also plotted in Figure two of the appendix. It is apparent that the effect of the sugar concentration is small between 100 and 200 g/l. However, there is a significant variation between that of 50 and 100, most probably due to osmotic effects. Of the incubation solutions examined, none show favorable effects for any sample. This is possibly due to too much of an osmotic pressure or perhaps too long of an incubation.

Study number five is done on standard 2% glucose agar. The variables in this study are incubation medias and length of incubation (2 and 4 hrs).

As summarized in Table eight, glucose-100 incubation has the best effects followed by glucose-50 and fructose-100. Longer incubation times appear to decrease the regeneration value, this may be the reason for the overall decrease in regeneration from incubations.

TABLE EIGHT--REGENERATION STUDY #5

INCUBATION MEDIA	REGENERATION PERCENTAGE 2 hrs	 4 hrs
Zero control	9.5	
Glucose 50	2.3	1.2
Glucose 100	6.9	5.9
Fructose 100	1.6	0.8

There were three samples per value.

The final regeneration study repeats variables from previous studies and also explores the effects of temperature. Three medias used are standard glucose, standard fructose, and fructose-yeast. The incubation medias are as shown in the table, and the three temperatures of 15, 19, and 25 degrees Celsius are compared in Table nine. These values indicate a positive effect from incubation at one and two hours and confirms previous data.

TABLE NINE--REGENERATION STUDY #6

INCUBATION MEDIA	REGENERATION PERCENTAGES Standard Glucose
None (Control) @ 25C	21.4
None (Control) @ 19C	22.1
None (Control) @ 15C	25.4
Glucose Only, 1 hr	22.8
Glucose Only, 2 hr	28.2
Glucose 100, 1 hr	29.8
Glucose 100, 2 hr	31.0
Glucose-KCl-1, 1 hr	20.8
Glucose-KCl-1, 2 hr	19.8

For standard fructose plates, no incubation, 25C, regeneration= 9.9%
For fructose-yeast plates, no incubation, 25C, regeneration = 21.2%
There are four samples per value.

General conclusions that can be drawn from this collection of data are that in all experiments, glucose regeneration is more favorable than fructose. Sugar concentration appears to not be an important factor after reaching the 100g/l concentration. The percentage of agar appears to be an important factor, apparantly, the softer the agar, the better the regeneration. The question of incubation media and time remains less clearly defined. It appears that incubation in glucose-100 is favorable for a span of 1-2 hrs while none of the other medias have proven effective.

The results do indicate that extended incubation tends to lower regeneration. Possibly the stability of the reversion depends upon the agar providing a solid complex.

The final variable, temperature, has some effect upon regeneration. The 15 C sample had a better regeneration value, however, it grows significantly slower than the other samples and represents only a three percent improvement in study six. This indicates that unless regeneration is of utmost importance, keeping the plates at 15 C will not have the extra time loss offset by any significant gains. (See picture appendix for growth comparision at different temperatures.)

The regeneration values on the standard glucose plates have varied from preparation to preparation. The range spans from about 11% to highs at about 20%, without incubation or overlays at room temperature. Fructose values appear to lie in the range from 7% to about 10% on a standard plate. On the color plates, regeneration has been about 2% to 4%. Cells which are treated through the transformation protocol show a decrease in regeneration to about 0.9% on these plates.

A different regeneration study was the viability of protoplasts stored at four degrees Centrigrade. The cells were stored in isotonic and KCl buffers and plated every few days (two to four). The regeneration percentages were thereby calculated and the drop in viability recorded. Overall, the decrease was linear for about ten days and showed some signs of trailing off around the thirteenth day at which the viability had dropped to about 30% of the original. The decrease per day was about 7%. (Data is plotted out in Figure three)

The last study was on the effects of UV light on the regeneration of the cells. The drop was dramatic. After an one minute exposure, the regeneration dropped to 1.4% of the original untreated and decreased to as little as 0.05% in eight minutes. The data from this experiment is plotted in Figure four.

Transformation and Screening:

Four complete attempts at transformation and screening have been completed, the typical time span of each experiment being in the range of three to four weeks. The exact transformation procedure used varies from each experiment and are summarized in the following Table ten.

TABLE TEN--TRANSFORMATION CONDITIONS

EXPT. #	#of cells	μg DNA	total volume (μl)**	incubation media & time	regen %
1	2×10^5	0.42	23.3	Glu-KCl-1/1.5 hr	0.90
2	1×10^6	0.50	25.0	Glu-KCl-1/ 3 hr	0.00*
3	1×10^6	1.00	25.0	Glu-100/ 2 hr	0.83
4	2.5×10^5	100.00	100.0	Glu-100/1.25 hr	16.2

*regeneration was very poor, only 258 colonies regenerated out of an expected 10,000.

** total volume refers to initial DNA, cells, and 1/4 volume PEG solution for the first twenty minutes of the transformation on ice.

Screening showed no colonies which did not develop the blue colors. There were several problems with both methods of screening used. For transformations one through three, the color fructose media was used as the detection screen. Problems with this screen involved mostly the diffusivity of the bromophenol blue in the agar so that if more than thirty colonies existed on the plate, it was very difficult to determine the exact source of the color. The color assay is not particularly sensitive so that colonies typically had to develop to about a 4 to 6 mm diameter before a very definite blue halo develops. This therefore restricts greatly the number of colonies that can be screened to the physical limitations of producing and processing so many plates.

In order to remove the uncertainity of which colony is actually producing the color change, the microtiter screen was attempted. The two advantages of this screen are the much higher regeneration values achieved on the glucose media and the separation of each colony into an individual well, thereby producing a very definite screen. The problem with this screen is naturally the time consumption required. The fungus is not difficult to pick and contamination for transferring 96 separate colonies into each plate is surprisingly not a problem. However, the colonies are by no means as easily transferred as bacteria and consequently it requires on the order of 45 to 60 minutes per plate. Additionally, screening these plates takes in the range of 30 minutes so that total process time for a 96 colony screen is about 90 minutes.

Another problem in the transfer is the lack of growth in some of the microtiter wells. The colonies are moved while very small into about 250µl of fructose media. However, it is not possible to shake these plates. Due to the lack of effective aeration, several colonies did not grow (21.8%). The impossibility of screening the large number of colonies most likely required to find the transformant makes this method ineffective.

The final screen with iodide shows a little promise. The triiodide ion does appear to be produced. However, the C. fumago appears sensitive only at a very young stage. As the colonies become larger, the triiodide does not seem to have much effect besides causing the C. fumago to grow in more of a vertical position as opposed to spreading.

Screening therefore remains the major problem presently in the isolation of the mutation. In experiment 1, a total of 1034 were screened positively, 258 for experiment two, all regenerated colonies examined. For experiments three and four, 3213 out of 8241 total and 1127 out of 40,200 (microtiter screen) were examined. From this, one can only conclude that this particular transformation is not particularly efficient or common which is not unexpected. The various screens are pictured in the appendix.

Concluding Remarks:

Although no specific chloroperoxidase negative mutation has been isolated, the possibilities of transforming C. fumago remains good. Protoplasts can be produced in abundance and isolated. Although regeneration in this system is lower than that of others, percentages around 30% have been obtained.

From all the studies done, it appears that the best way to form, recover and regenerate protoplasts is as follows:

1) Harvest mycelia and resuspend into isotonic buffer.
2) Grind into an even suspension (about 2 minutes on homogenizer).
3) Digest with NovoZym 234 at 15mg/g mycelia, about three hours.
4) Harvest by centrifugation, resupend cells into $MgSO_4$ buffer, and band with an overlay of KCl buffer. Collect cells and concentrate by by centrifugation and resupension into desired buffer.
5) To regenerate, incubate cells in Glucose-100 solution, about 1 hour.
6) For the highest regeneration, plate cells in overlay of 0.8-1.0% glucose agar onto the standard glucose plates (these plates have to be around 2% agar or else it is too soft to plate onto). The plates can then be stored at 15 C after sitting out overnight. If regeneration is not of the utmost importance, the cells can be plated directly after incubation and kept at room temperature where they will grow much faster.

Since the above procedure is fairly well worked out, the problem that presently exists is the screening for the mutation. The attempted very specific type III integration is rare. Therefore, a selection capable of handling up to 10^8 cells is needed. Two possible methods exist. Either a selection marker is needed or a screen capable of killing all chloroperoxidase active cells must be developed.

The selection marker is presently being developed by William Patterson in our laboratory which would involve the insertion of a hygromycin resistance gene into the pUCCF6 . Although hygromycin does not kill untransformed C. fumago, it inhibits growth significantly when the protoplasts are grown in "liquid" plates and maintained at 15 degrees Centigrade. "Liquid" plates involve spreading the growth media with cells in a very thin layer across the bottom of the plates so that the regenerated cells grow by adhesion to the bottom. The development of these plates was necessitated by the ineffectiveness of the hygromycin in agar. C. fumago can grow in agar containing 1 mg/ml hygromycin. In the liquid plates developed by Mr. Patterson, the hygromycin is effective at 400µg/ml. Therefore, this system appears promising.

An added advantage to this selection method is that it may potentially show whether the DNA has or has not been taken into the cell. If abortive transformants arise without the presence of stable colonies, this will show at least that DNA is indeed taken into the cell and perhaps partially integrated. If stable transformants do occur, these can be screened again for the presence of chloroperoxidase. This will greatly enrich for the desired mutant and the color screen can thus be used effectively within a reasonable time span (28).

The second possible screen is based on "suicide". This would involve placing a compound in the agar that can be halogenated by the chloroperoxidase. The halogenated form would be toxic so that absorption by the colony would result in its death. The results from using iodide give mixed results. Some colonies were indeed killed off by the triiodide formation, however other colonies surviving past what appears to be the critical size grew quite well, albeit slower than normal and darker.

This screen does appear to have some potential, but it needs to be worked out quite a bit. Perhaps the greatest setback here is the diffusivity of the formed toxic triiodide ion as this would tend to kill off all cells. The only apparant solution to this would be to somehow fix the triiodide so that very little diffusion occurs or to produce the ion very early, almost at the microscopic stage so that very little excess builds up in the media. From the pilot experiment, it does appear that active chloroperoxidase is produced at a very young state, when the fungus is just visible on the agar. This, however, may be too late of a stage to allow effective screening of many cells, at least in the order of 10^3/plate.

Besides the screening, another problem with the transformations may be with the actual method used. Polyethlyene glycol is typically used to cause cell fusion, a highly undesirable result in this particular experiment. It may therefore be necessary in this case to resort to high tech methods such as DNA injection or the older method used with plant cells, electroporation.

Despite the present problems, the development of C. fumago as an expression vector is quite feasible. If the chloroperoxidase negative mutation is too difficult to isolate due to the need of preventing any cell fusion and knocking out all gene copies present in the protoplast, transformation can still be carried out with a selection marker. The gene system can still be used to express engineered proteins. As the C. fumago shows good potential as an industrial organism for biotechnology, further investigations into this system are warranted.

APPENDIX

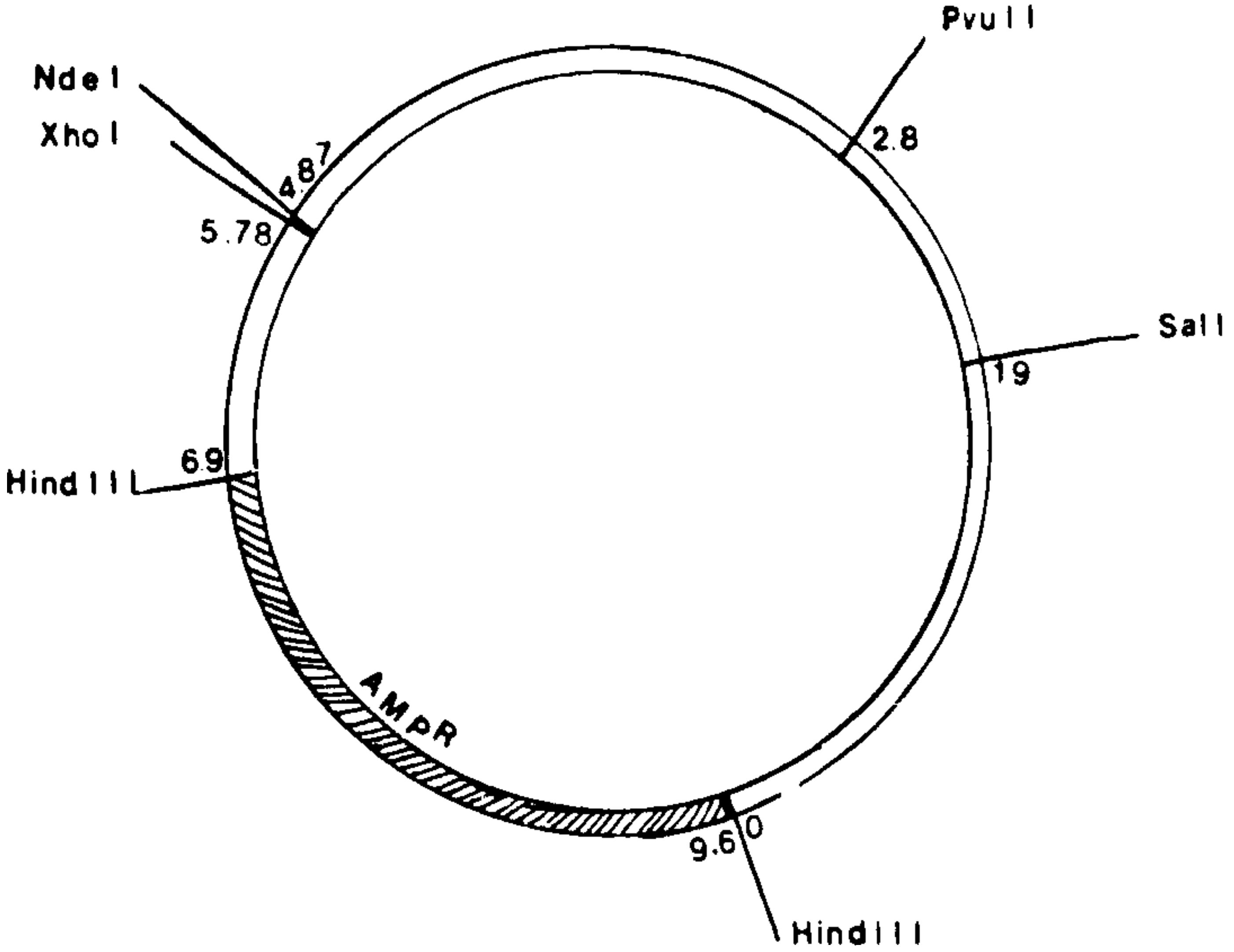


FIGURE ONE MAP OF pUCCF6▲

▨ Plasmid
☐ Insert
■ Polylinker

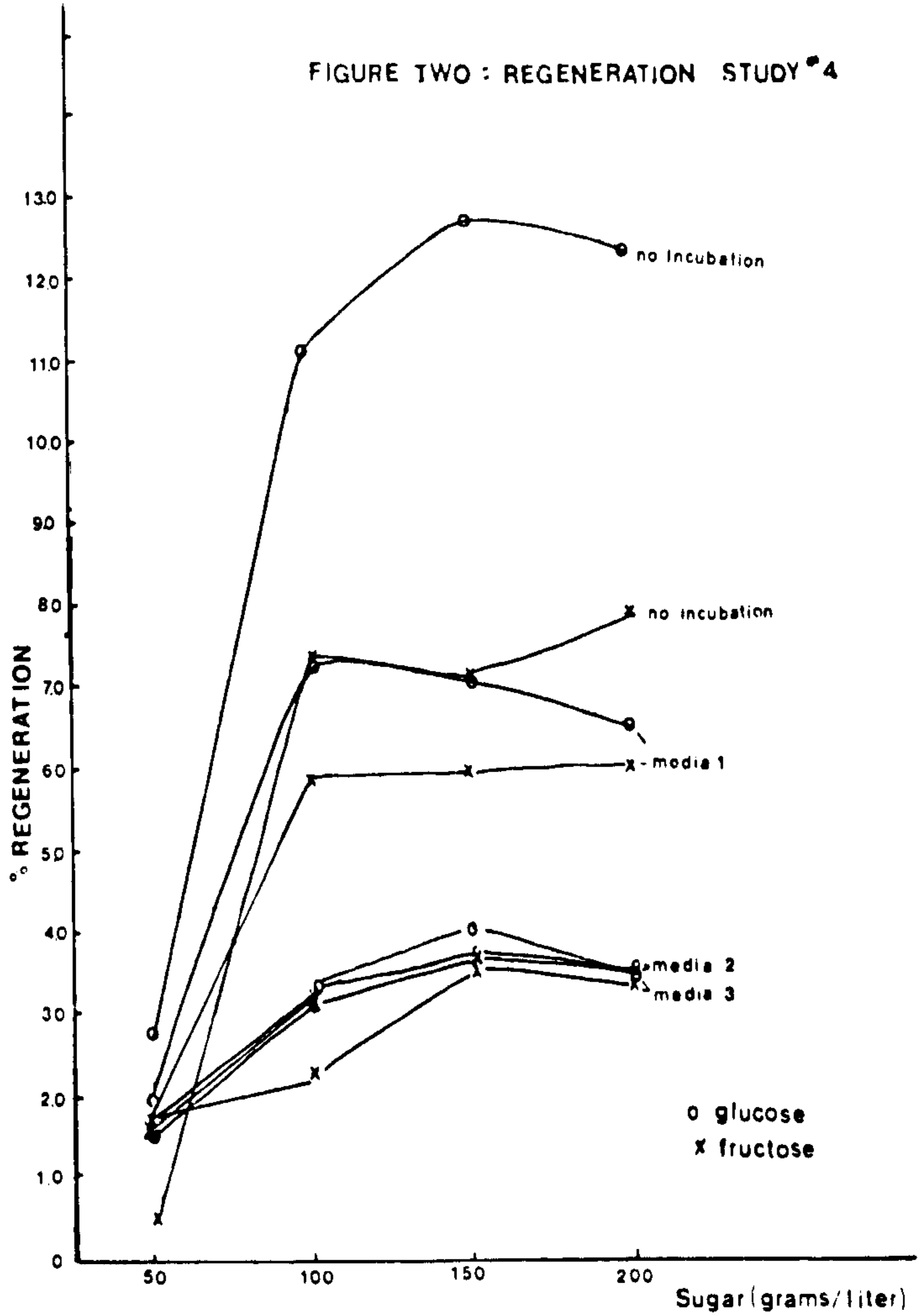


FIGURE TWO : REGENERATION STUDY #4

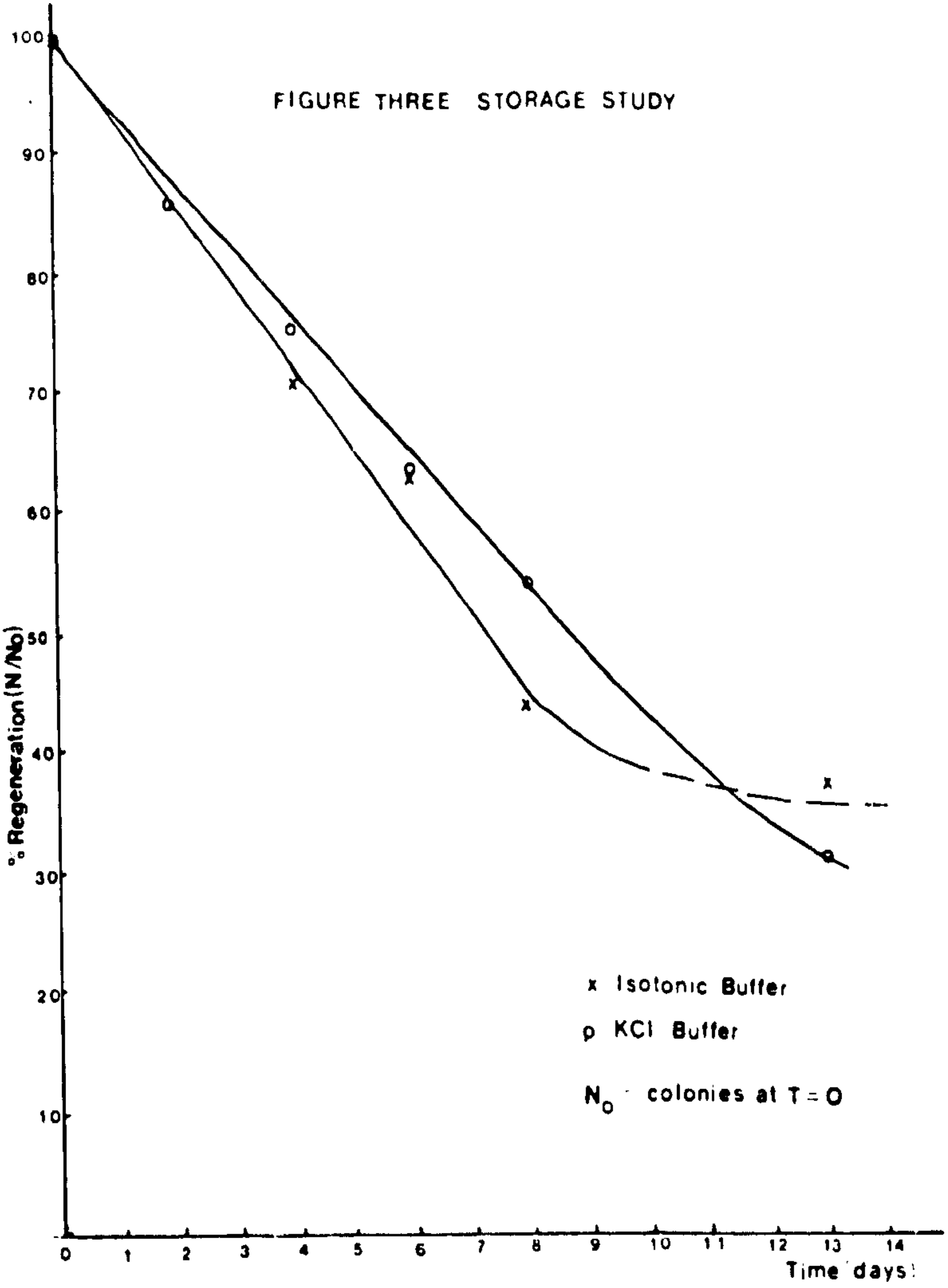


FIGURE THREE STORAGE STUDY

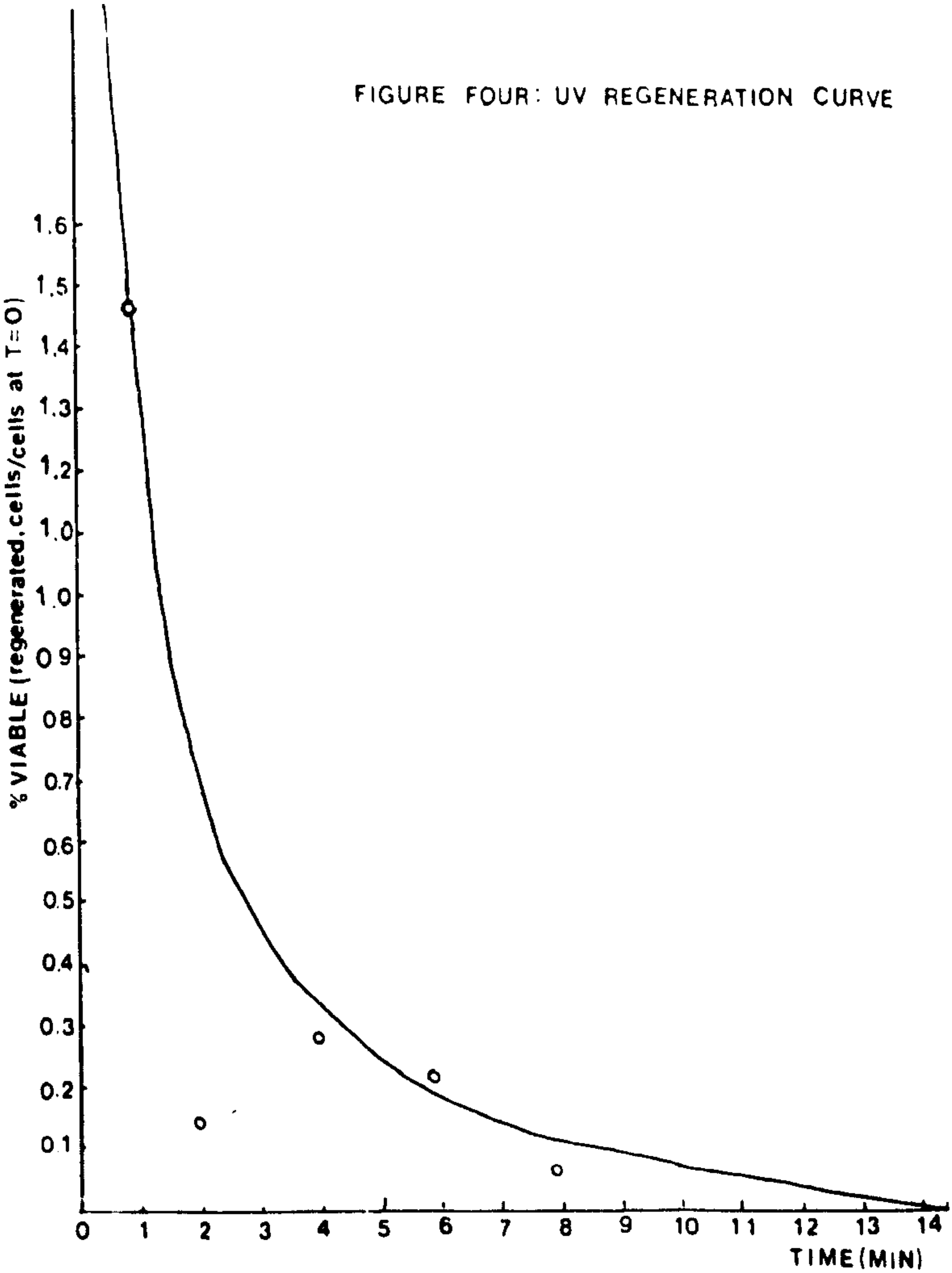
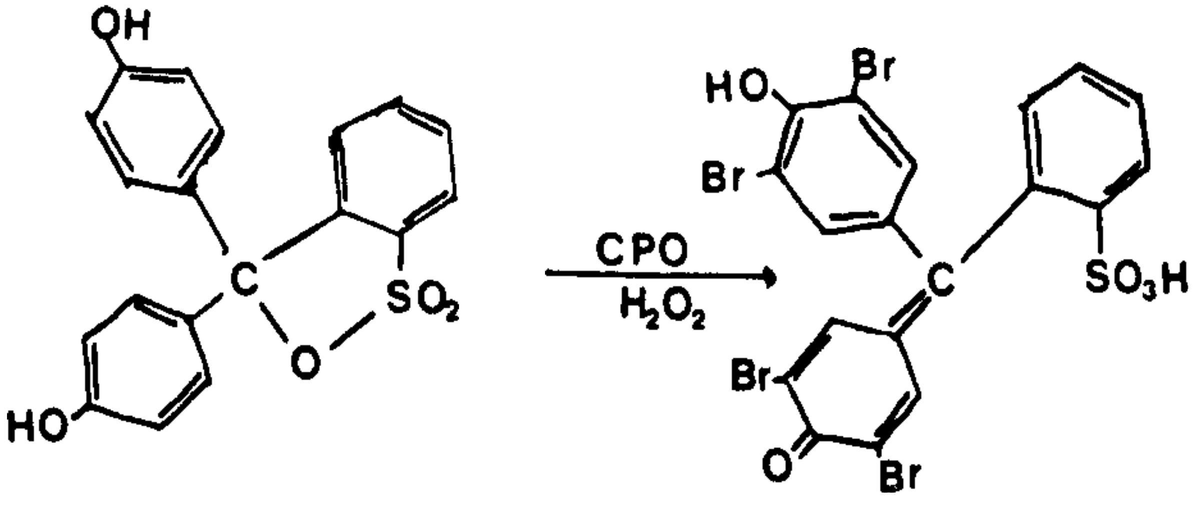


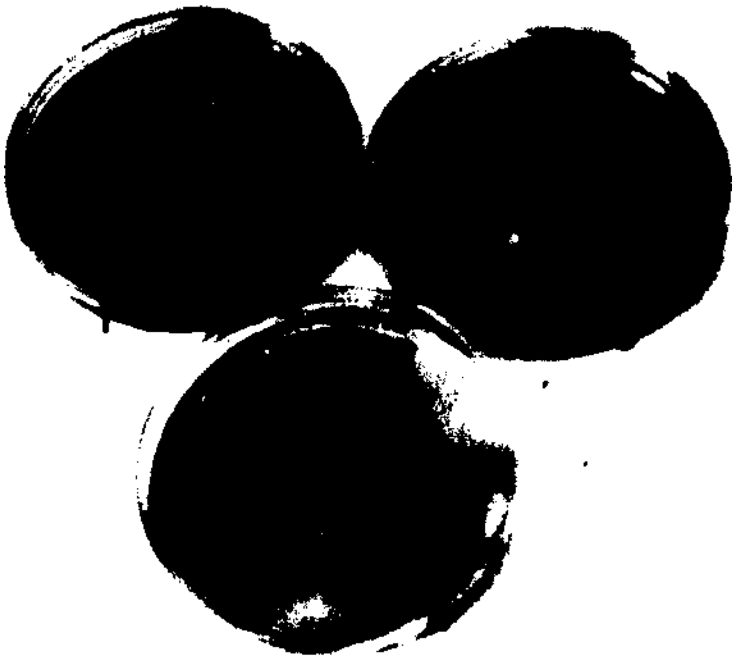
FIGURE FOUR: UV REGENERATION CURVE

FIGURE FIVE-COLOR ASSAY



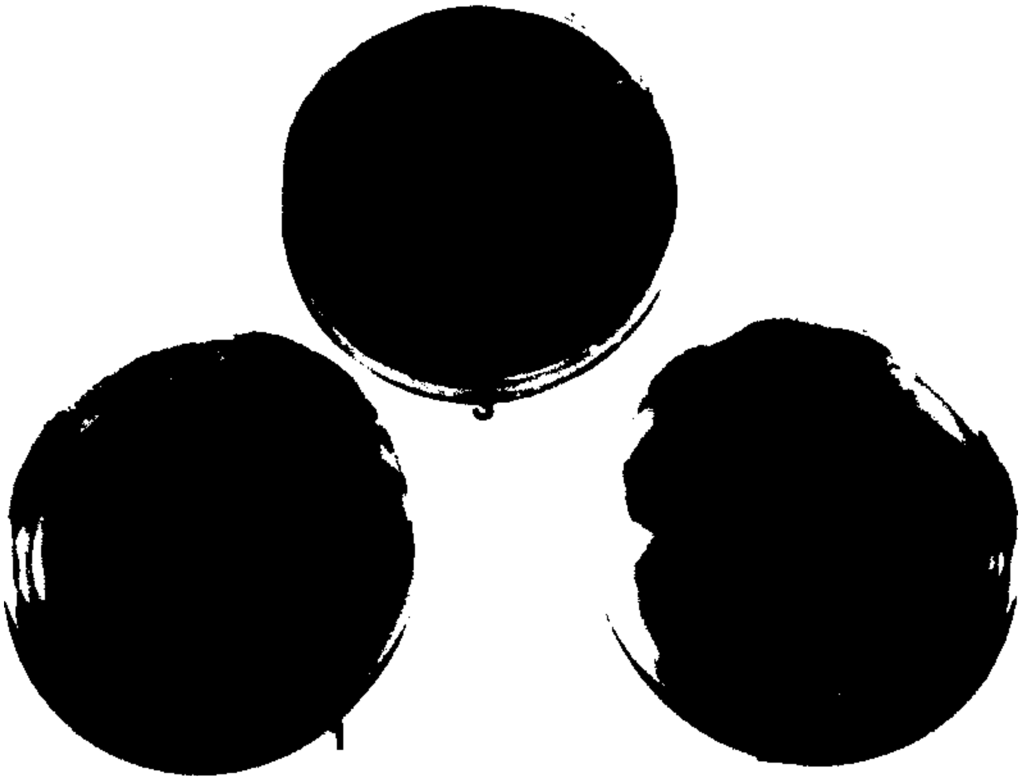
PHENOL RED

BROMOPHENOL BLUE



PICTURE ONE
--growth medias, 1 wk

1 Glucose/Malt
2 Fructose/Malt
3 Fructose/Yeast



PICTURE TWO
-- 2 Weeks Growth
Same legend as one

PICTURE THREE--TEMPERATURE COMPARISION

1 -- 15 C

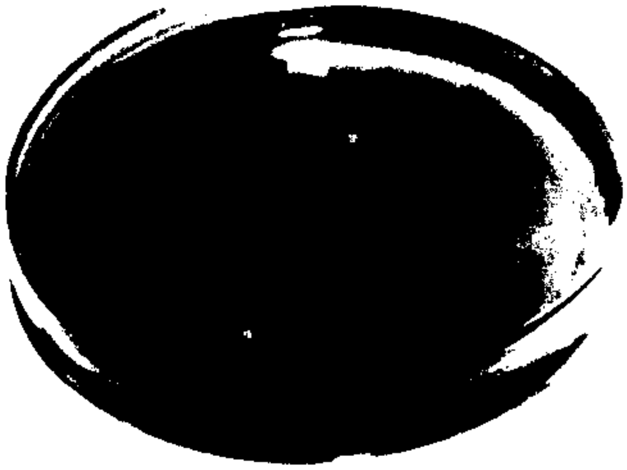
2 - 19 C

3 -- 25 C

PICTURE FOUR

GLUCOSE/MALT

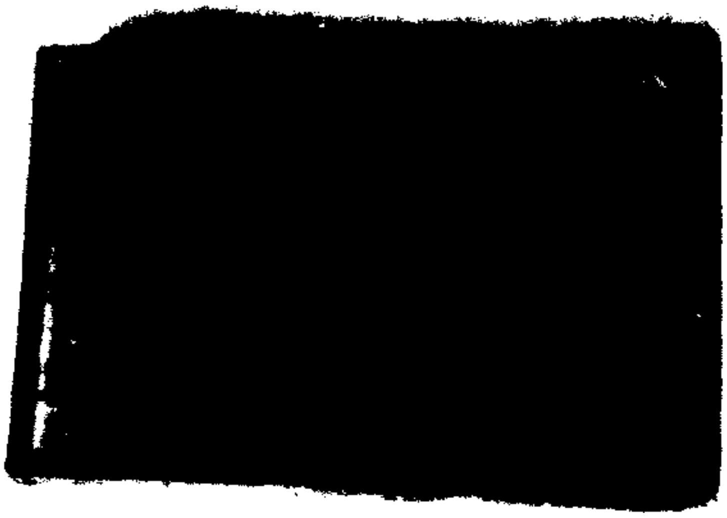
GROWTH, 2 Wks



PICTURE FIVE

COLOR ASSAY

IN AGAR



PICTURE SIX -

COLOR DETECTION

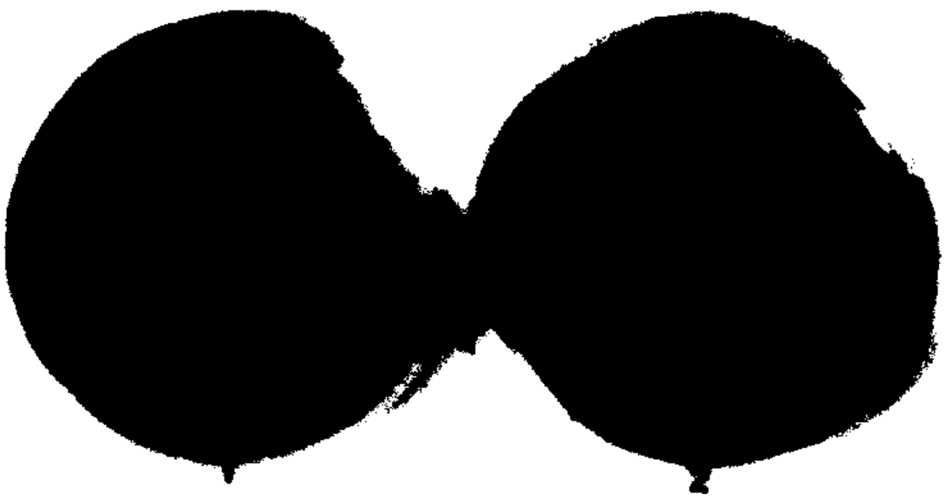
IN MICROTITER DISH

PICTURE SEVEN

IODINE SCREEN

[illegible]

[illegible]



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